

## Research Note

# Differential Expression of Cardiac Muscle Mitochondrial Matrix Proteins in Broilers from Ascites-Resistant and Susceptible Lines<sup>1</sup>

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**ABSTRACT** Ascites is a metabolic disorder of modern broilers that is distinguished by cardiopulmonary insufficiency in the face of intense oxygen demands of rapidly growing tissues. Broilers with ascites exhibit sustained elevation of pulmonary arterial pressure and right ventricular hypertrophy, the end result of which is heart failure. It has been shown that mitochondrial function is impaired in broilers with ascites. In the current study, mitochondrial matrix protein levels were compared between ascites-resistant line broilers and ascites-susceptible line broilers with and without ascites using two-dimensional (2-D) gel electrophoresis. One hundred seventy-two protein spots were detected on the gels, and 9

of the spots were present at different levels in the 4 groups of broilers. These 9 protein spots were selected for identification by mass spectrometry. Two of the spots were found to contain single mitochondrial matrix proteins. Both mitochondrial matrix proteins, the dihydrolipoamide succinyltransferase component of the 2-oxoglutarate dehydrogenase complex and the  $\alpha$ -subunit of mitochondrial trifunctional enzyme, were present at higher levels in ascites-resistant line broilers with ascites in the present study. The elevated levels of 2 key proteins in aerobic metabolism in ascites-resistant line broilers with ascites observed in the present study suggests that the mitochondria of broilers with this disease may respond inappropriately to hypoxia.

(Key words: ascites syndrome, cardiac muscle, mitochondria, proteome, pulmonary hypertension syndrome)

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## INTRODUCTION

Ascites syndrome is a metabolic disease of broilers characterized by pulmonary hypertension-induced right ventricular hypertrophy and failure (Julian, 1993, 2000; Wideman, 2001). Genetic selection has been used to create ascites-resistant and ascites-susceptible broiler lines. The data from these studies indicate that a small number of major genes are probably involved in resistance to ascites in broilers (Shlosberg et al., 1996; Wideman and French, 1999, 2000; Anthony et al., 2001; Anthony and Balog, 2003; Balog, 2003; Balog et al., 2003). It has been shown that mitochondrial function is defective in broilers with ascites (Cawthon et al., 1999, 2001; Iqbal et al., 2001a,b; Tang et al., 2002). In addition, it has also been observed that mitochondrial electron transport chain proteins in cardiac muscle were elevated in ascites-resistant line broilers

without ascites raised under simulated high altitude conditions (Cisar et al., 2004). The objective of the present study was to examine cardiac mitochondrial matrix proteins of ascites-resistant and ascites-susceptible line broilers and determine if additional mitochondrial proteins are differentially expressed in the genetically selected lines.

## MATERIALS AND METHODS

### **Broiler Lines, Induction of Ascites, and Collection of Cardiac Tissues**

The ascites-resistant and ascites-susceptible broiler lines used in this study have been described previously (Anthony et al., 2001; Anthony and Balog, 2003; Balog, 2003; Balog et al., 2003; Cisar et al., 2004). Sire-family selection for resistance and susceptibility to ascites has been applied over several generations. Progeny are reared for 6 wk in a hypobaric chamber under simulated high altitude conditions (2,900 m above sea level) to induce ascites (Odom et al., 1992; Balog et al., 2000). Ascites

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**Abbreviation Key:** 2-D = two-dimensional; FADH<sub>2</sub> = flavin adenine dinucleotide reduced; NADH = nicotinamide adenine dinucleotide; RV:TV = right ventricle to total ventricle weight ratio.

TABLE 1. Line and disease status of broilers used in study

Line <sup>1</sup>	Ascites <sup>2</sup>	RV:TV <sup>3</sup>
Resistant	Normal	0.247 ± 0.021
	Ascites	0.459 ± 0.021
Susceptible	Normal	0.283 ± 0.025
	Ascites	0.418 ± 0.025

<sup>1</sup>The 2 broiler lines used in this study were ascites resistant (n = 6; 3 normal, 3 ascitic) and ascites susceptible (n = 4; 2 normal, 2 ascitic). Chicks were raised in a hypobaric chamber simulating high altitude (2,900 m above sea level) for 20 to 21 d.

<sup>2</sup>Birds were classified as normal if they were asymptomatic and had a right ventricle to total ventricle weight ratio (RV:TV) ≤ 0.313. Birds were classified as having ascites if fluid was present in the abdominal cavity and their RV:TV ≥ 0.408.

<sup>3</sup>Data are means ± SE.

mortality data are then used to select the most resistant and susceptible sire families for reproduction of the lines. In the ninth generation the ascites-resistant line exhibited 6% ascites mortality, and the ascites-susceptible line exhibited 80% ascites mortality under simulated high altitude conditions (2,900 m above sea level).

For the current study, chicks from ninth generation ascites-resistant and susceptible broiler lines were placed at 1 d of age in a hypobaric chamber at a simulated altitude of 2,900 m above sea level (Balog et al., 2000). The broilers were housed in stainless-steel battery units equipped with nipple waterers and fed ad libitum. Heart tissues were harvested from 20- to 21-d-old birds, divided into right ventricle and left ventricle plus septum, and weighed. Right ventricle weight to total ventricle weight ratios (RV:TV) are indicative of the severity of pulmonary hypertension (Burton et al., 1968) and were used in conjunction with physical symptoms to classify birds with or without ascites. Broilers with ascites had RV:TV ≥ 0.4 and fluid was present in the abdominal cavity. Broilers without ascites had RV:TV ≤ 0.3 and were asymptomatic. A description of the ascites-resistant and susceptible line broilers used in the present study is shown in Table 1.

### Preparation and 2-D Gel Electrophoresis of Mitochondrial Protein Samples

Mitochondria were isolated from freshly harvested cardiac right ventricle tissues by protease digestion of the tissue followed by homogenization and differential centrifugation using a mitochondria isolation kit (MITO-ISO1).<sup>3</sup> A cocktail of protease inhibitors (P8340)<sup>3</sup> was added to the mitochondria and individual samples were stored at -80°C.

Mitochondrial protein samples were prepared for 2-D gel electrophoresis using a PlusOne 2D Clean-Up Kit<sup>4</sup>

and were resuspended in isoelectric focusing buffer (8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% pH 3-10 carrier ampholytes, 40 mM dithiothreitol, and 0.002% bromophenol blue). Protein concentrations were determined independently for each bird mitochondrial preparation using a PlusOne 2D Quant Kit.<sup>4</sup> Two-dimensional gel electrophoresis was performed using the ZOOM IPGRunner system according to the manufacturer's instructions.<sup>5</sup> For the first dimension 45 µg of mitochondrial protein were separated for 2,600 V·h on pH 3-10 NL ZOOM IPG strips<sup>5</sup> using a step voltage program (0 to 2,000 V) with a final focusing step of 2,000 V for 30 min. Second dimension gel electrophoresis was performed using 4 to 12% Bis-Tris ZOOM gels.<sup>5</sup> Gels were electrophoresed for 85 min at 150 V. Gels were stained with SYPRO Ruby Protein Gel Stain<sup>5</sup> according to the manufacturer's instructions. Proteins were detected using a Typhoon 8600 laser scanner.<sup>4</sup>

### Data Analysis and Protein Identification

Protein spots were detected automatically and quantified with a computer program, Melanie 4.<sup>6</sup> Protein quantities are reported as percentage volumes in which volume is based on pixel intensity and is the volume above the spot border at 75% of spot height (measured from the peak of the spot). Percentage volume refers to the relative volume of an individual spot and is calculated by dividing an individual spot volume by the sum of all spot volumes for that gel. Statistical methods included central tendency and dispersion for spot values and overlapping measures (quantification of the overlap between spot values between classes). Differentially expressed proteins were selected from the polyacrylamide gels and sent to Protana Analytical Services<sup>7</sup> for identification by mass spectrometry (MALDI-TOF MS/MS).

## RESULTS AND DISCUSSION

Several studies have shown that mitochondrial function is defective in a variety of tissues in broilers with ascites (Cawthon et al., 1999, 2001; Iqbal et al., 2001a, b; Tang et al., 2002). Cisar et al. (2004) showed previously, using quantitative Western blots, that several mitochondrial electron transport chain proteins were present at elevated levels in right ventricle cardiac muscle of broilers from an ascites-resistant line that did not develop ascites under simulated high altitude conditions. In the current study, the mitochondrial proteomes of right ventricular cardiac muscle from the same ascites-resistant and -susceptible broiler lines exposed to similar conditions were examined and 172 protein spots were detected on 2-D gels. Analysis and comparison of the protein spots on the gels revealed that most of the mitochondrial proteins were present at similar levels in the 4 groups (ascites-resistant line broilers with and without ascites and ascites-susceptible line broilers with and without ascites). Nine proteins, however, were present at different levels in the four groups. These nine proteins were selected for additional

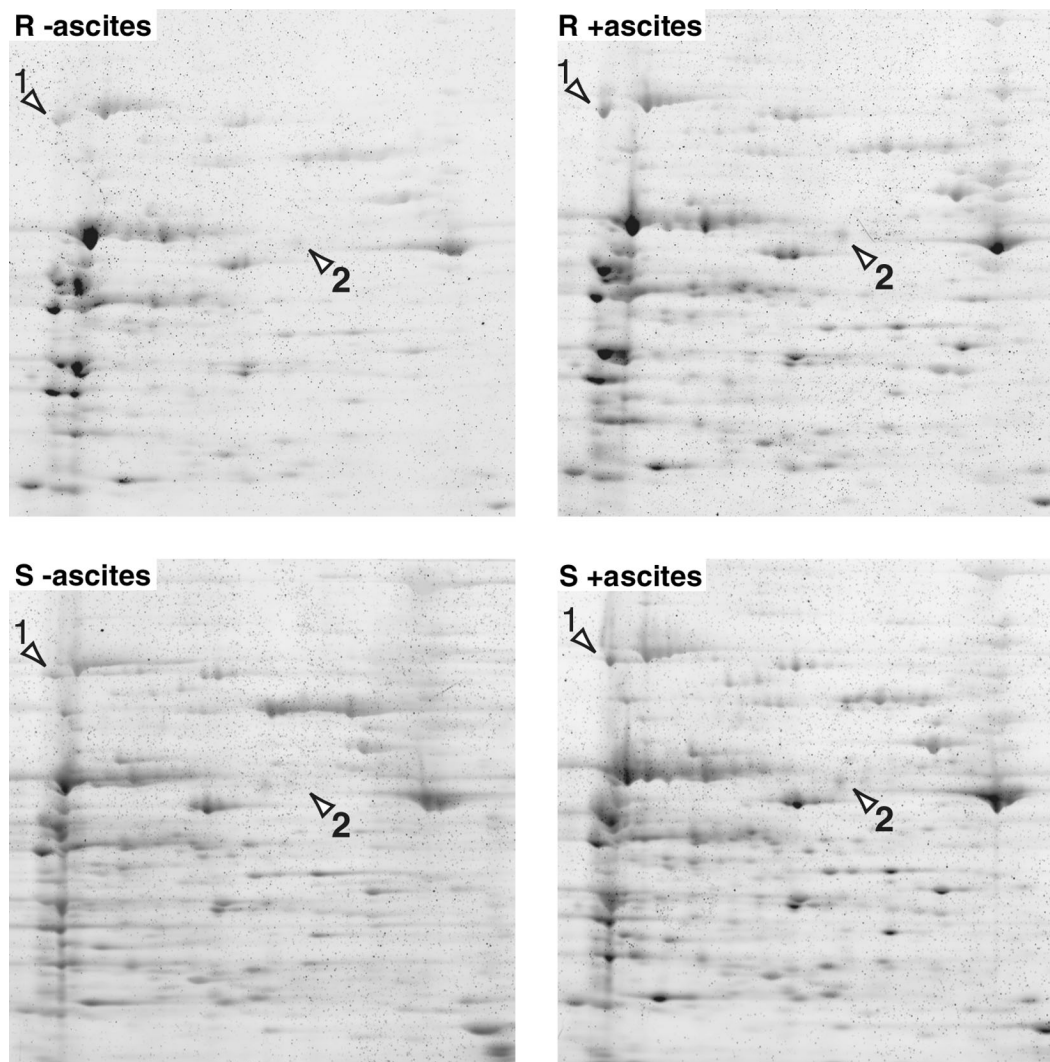
<sup>3</sup>Sigma, St. Louis, MO.

<sup>4</sup>Amersham Biosciences, Piscataway, NJ.

<sup>5</sup>Invitrogen Corporation, Carlsbad, CA.

<sup>6</sup>Geneva Bioinformatics (GeneBio) S.A., Geneva, Switzerland.

<sup>7</sup>Protana Analytical Services, Toronto, Canada.



**FIGURE 1.** Representative 2-dimensional gels containing mitochondrial proteins from right ventricle cardiac muscle of broilers from ascites-resistant and susceptible lines are shown. Broilers were exposed to hypobaric conditions for 20 to 21 d. Four groups of broilers were compared: ascites-resistant line broilers with and without ascites (R + ascites and R – ascites, respectively) and ascites-susceptible line broilers with and without ascites (S + ascites and S – ascites, respectively). The arrow labeled (1) indicates the location of the alpha subunit of mitochondrial trifunctional enzyme and the arrow labeled (2) indicates the location of the dihydrolipoamide succinyltransferase component of the 2-oxoglutarate dehydrogenase complex.

analysis and their identities were determined by mass spectrometry. Two protein spots contained single mitochondrial matrix proteins (Figure 1; Table 2). The other seven spots contained multiple proteins, were mitochondrial membrane proteins (which cannot be reliably resolved and quantitated under the conditions used in the present study), or were non-mitochondrial proteins and, therefore, were not considered further.

The two mitochondrial matrix proteins identified by mass spectrometry were the dihydrolipoamide succinyltransferase component of the 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61) and the alpha subunit of mitochondrial trifunctional enzyme (EC 4.2.1.17 and EC 1.1.1.35). Both proteins were present at higher levels in ascites-resistant line broilers with ascites than in ascites-resistant line broilers without ascites or ascites-suscepti-

ble line broilers with or without ascites. Dihydrolipoamide succinyltransferase component of the 2-oxoglutarate dehydrogenase complex and  $\alpha$ -subunit of mitochondrial trifunctional enzyme levels were, respectively,  $1.79 \pm 0.38$ - and  $1.86 \pm 0.25$ -fold higher in ascites-resistant line broilers with ascites. Heart muscle depends almost entirely on aerobic metabolism, and fatty acids are its primary fuel source. Mitochondrial trifunctional enzyme is an important enzyme in the  $\beta$ -oxidation of fatty acids. This pathway produces nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide reduced ( $\text{FADH}_2$ ), and acetyl-coenzyme A, which is funneled into the citric acid cycle producing additional NADH and  $\text{FADH}_2$ . These cofactors, NADH and  $\text{FADH}_2$ , are required for oxidative phosphorylation. The enzyme complex, 2-oxoglutarate dehydrogenase, is a

TABLE 2. Expression of mitochondrial matrix proteins in broilers from ascites-resistant and ascites-susceptible lines

Protein <sup>1</sup>	Resistant line		Susceptible line	
	–Ascites <sup>2</sup> (% vol)	+Ascites <sup>2</sup> (% vol)	–Ascites <sup>2</sup> (% vol)	+Ascites <sup>2</sup> (% vol)
EC 2.3.1.61 <sup>3</sup>	0.2588 ± 0.0330	0.3548 ± 0.0330	0.1497 ± 0.0405	0.2170 ± 0.0405
EC 4.2.1.17/EC 1.1.1.35 <sup>4</sup>	0.4043 ± 0.0635	0.6017 ± 0.0635	0.2768 ± 0.0778	0.3141 ± 0.0778

<sup>1</sup>Proteins were identified by mass spectrometry (MALDI-TOF MS/MS). Identifications were based on amino acid sequence matches with 8 and 16 tryptic peptides for EC 2.3.1.61 and EC 4.2.1.17/EC 1.1.1.35, respectively.

<sup>2</sup>Broilers were from ascites-resistant and -susceptible lines. Broilers without ascites are designated –ascites and broilers with ascites are designated +ascites. Data are given as percentage volume (% vol) and are presented as means ± SE.

<sup>3</sup>Dihydrolipoamide succinyltransferase component of the 2-oxoglutarate dehydrogenase complex. Ensembl translation ID is ENSGALP 00000016718. Protein and gene information can be accessed at [www.ensembl.org/Gallus\\_gallus](http://www.ensembl.org/Gallus_gallus).

<sup>4</sup> $\alpha$ -Subunit of mitochondrial trifunctional enzyme (also known as CFR-associated protein p70 and gastrin binding protein). Ensembl translation ID is ENSGALP 00000026633. Protein and gene information can be accessed at [www.ensembl.org/Gallus\\_gallus](http://www.ensembl.org/Gallus_gallus).

key control site in the citric acid cycle. The cycle rate is regulated by feedback inhibition of 2-oxoglutarate dehydrogenase. Under the hypoxic conditions experienced by tissues in broilers with ascites, aerobic respiration is usually inhibited thereby reducing mitochondrial function (Schumacker et al., 1993; Duranteau et al., 1998). The observation that key mitochondrial matrix proteins involved in aerobic metabolism are specifically elevated in ascites-resistant line broilers with ascites suggests that the mitochondria of these birds may not respond appropriately to hypoxic conditions.

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